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Research Article

Toxicity of Aqueous Fullerene nC_{60} to Activated Sludge: Nitrification Inhibition and Microtox Test

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The increasing production and use of fullerene nanomaterials raised their exposure potential to the activated sludge during biological wastewater treatment process. In this study, the toxicity of aqueous nanoscaled C_{60} (nC_{60}) to activated sludge was investigated using nitrification inhibition and Microtox test. The test solutions of nC_{60} were prepared using two methods: long stirring (Stir/nC_{60}) and toluene exchange (Tol/nC_{60}). The nC_{60} aggregation in test medium was also evaluated for toxicity assessment. The results showed that the nC_{60} aggregation behaved differently in two test mediums during the incubation periods. The nC_{60} toxicity was greatly influenced by the preparation method. Stir/nC_{60} presented no significant toxicity to both the nitrification sludge and bioluminescent bacteria at the maximum concentration studied. In contrast, the EC_{20} of Tol/nC_{60} was obtained to be 4.89 mg/L (3 h) for the nitrification inhibition and 3.44 mg/L (30 min) for Microtox test, respectively.

1. Introduction

Fullerenes, carbon-based nanomaterials, are demonstrating rapid increase in the commercial and scientific interest by their unique properties, such as, chemically and thermally stable, excellent electron acceptor and radical scavenger, and special optical properties [1, 2]. The production is expected up to 1500 t in 2007 compared to 400 kg in 2002 by the largest fullerene production company in the world [3]. In addition, until 2011 the class of fullerenes and other carbon-based nanomaterials is ranked as the second among all the nanomaterials used in consumer products available [4].

The C_{60}, the main type of fullerenes, showed the toxicity on cell [5], bacterial [6, 7], and fish [8, 9]. Although the toxicity mechanism is still not clear, the most published is due to the oxidative stress via reactive oxygen species dependent [5] or independent [10]. The toxicities suggested the potential of adverse effect of C_{60} on the activated sludge which is important for the removal of organic matters and nutrient compounds in wastewater. Recent studies pointed out the toxicity of metal nanoparticles (silver [11, 12], copper [13], zinc oxide, and titanium dioxide [14]), on the aerobic/anaerobic activated sludge. However, very limited studies focused on fullerene C_{60} toxicity on the activated sludge. Kang et al. [15] reported about 30% inactivation of microorganism in wastewater samples after 1 h exposure to nC_{60}-coated filter. However, no significant change was identified to microbial community structure in the anaerobic sludge using the denaturing gradient gel electrophoresis analysis under 50 g/kg biomass [16].

The objective of this study is to assess the toxicity of aqueous nC_{60} on the activated sludge. The effect of nC_{60} on nitrification activities was investigated using the cultivated activated nitrifying sludge. The Microtox test was also conducted as a standardized screening test. To our best knowledge, this is the first study on the nC_{60} toxicity on activated sludge by checking the nitrification inhibition. This work is expected to provide useful information to assess the effect of nC_{60} on activated sludge and consequent treatment performance of biological wastewater treatment process.

2. Materials and Methods

2.1. Preparation of Aqueous nC_{60} Suspension. Two types of aqueous nC_{60} were prepared using the extended mixing of

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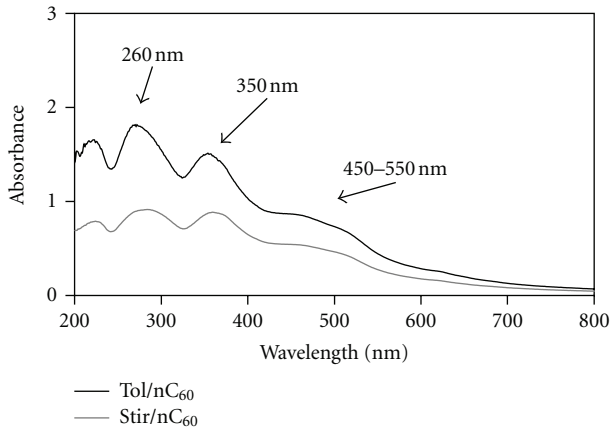

Figure 1: UV-visible absorption spectra of prepared nC_{60} (pH 5.6).

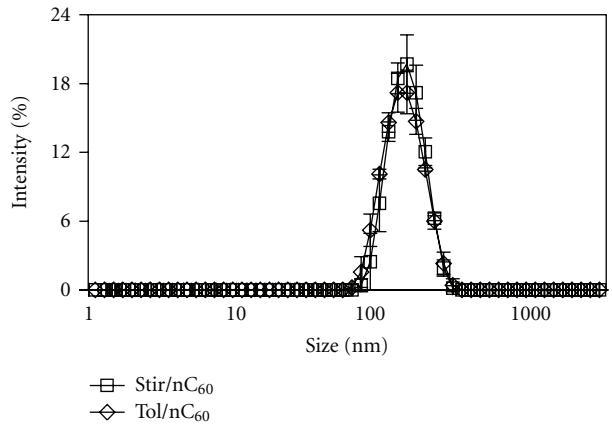

Figure 2: Size distribution of prepared nC_{60}. Error bars represent the standard error (pH 5.6, $n = 2$).

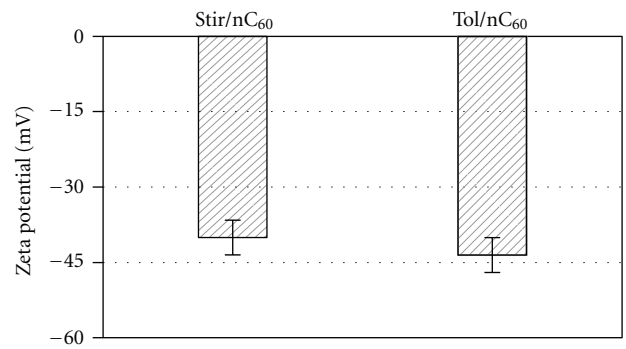

Figure 3: Zeta potential of prepared nC60. Error bars represent the standard error (pH 5.6, $n = 2$).

powder C_{60} in water (Stir/nC_{60}) and the toluene-involved solvent exchange method (Tol/nC_{60}), both of which are widely used for the studies of the C_{60} toxicity [7, 9, 15] and its fate [17, 18]. Briefly, the Stir/nC_{60} was produced by adding 200 mg of powder C_{60} (purity: 99.9%, SES research, USA) to 300 mL of ultrapure water (Millipore, USA) and then mixing with a magnetic stirrer at 500 rpm for three weeks. And the Tol/nC_{60} was prepared by following the previously reported method with minor modifications [7]. Specifically, 1000 mg/L C_{60} solution was obtained by dissolving the powder C_{60} into HPLC grade toluene (stock C_{60} toluene solution). And then 40 mL of the solution was added to 100 mL ultrapure water. The toluene was removed by sonication at 40°C using an ultrasonic cleaner (AS ONE, Japan) followed by the purge with gentle stream of nitrogen gas at 0.5 L/min for 1 h. The prepared Stir/nC_{60} and Tol/nC_{60} suspensions were, both of yellow colour, sequentially filtered through a glass filter (pore size: 1 μm, Pall Life Sciences, USA) and cellulose acetate filter (pore size: 0.45 μm, Advantec, Japan). In addition, a blank sample for Tol/nC_{60} was also prepared by adding the same amount of toluene (without C_{60}) in the pure water followed by the same procedures of sonication and filtrations. The resulting suspensions were stored in the dark at 4°C until use. The nC_{60} concentration was determined by extracting nC_{60} into the toluene phase and quantifying it at 332 nm by the UV/vis spectrometer, described elsewhere [7, 19, 20]. The calibration curve was obtained by the stock C_{60} toluene solution at different concentrations ($n = 5$).

2.2. Cultivation of Nitrifying Activated Sludge. The seed-activated sludge was collected from the nitrification tank of a biological wastewater treatment plant. The nitrifying activated sludge was obtained by cultivating the seed sludge at 30°C in a 3 L water-jacketed glass reactor. The reactor was operated at fill and draw mode at a hydraulic retention time of 12 h and sludge retention time of 20 d in the dark. Dissolved oxygen was kept at above 1.0 mg/L by introducing the filtered compressed air via diffusers, and the pH was maintained at 7.5 ± 0.1 with the automatic addition of 1 M Na_2CO_3. The feed solution only consisted of the inorganic medium with the 25 mM $(NH_4)_2SO_4$. The other nutrient composition, data acquisition, and process control were described in the previous study [21]. After reaching the steady state by checking the NH_4^+ removal efficiency, the mixed liquor in the reactor was collected and washed three times using 40 mM KH_2PO_4 buffer (pH 7.8) by centrifugation (2000 g × 5 min).

2.3. Nitrification Inhibition Experiment. The nitrification inhibition studies were conducted in accordance with the ISO 9509 test guideline [22]. Batch experiments were carried out by agitating 100 mL Erlenmeyer flasks containing 50 mL medium solution (2 mM $(NH_4)_2SO_4$ and 6 mM $(NaHCO_3)$), determined nitrifying activated sludge and different amounts of nC_{60} at controlled room temperature (25°C). The experimental conditions, the incubating time of 3 h and MLSS of 40 mg/L, were determined to ensure about 50% of initial NH_4^+ that was left at the end of incubating time to avoid rate limiting [22]. The dissolved oxygen was kept at above 4 mg/L by the shaking (150 rpm) on a rotary shaker. The nitrification inhibition (I) was calculated by the difference of oxidized N

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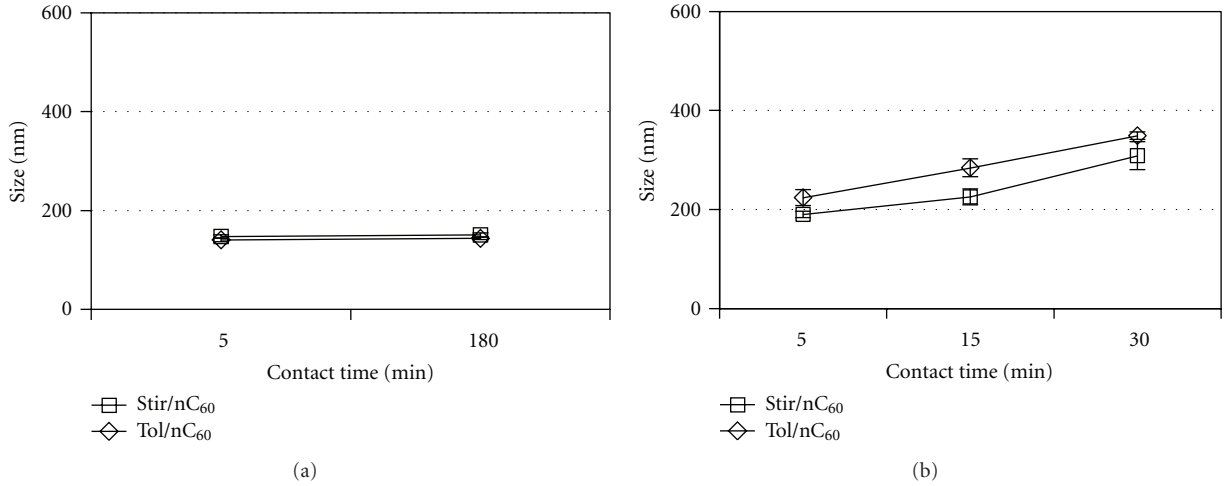

FIGURE 4: Change in nC_{60} size in medium for nitrification inhibition (a) and Microtox test (b). Error bars represent the standard error ($n = 2$).

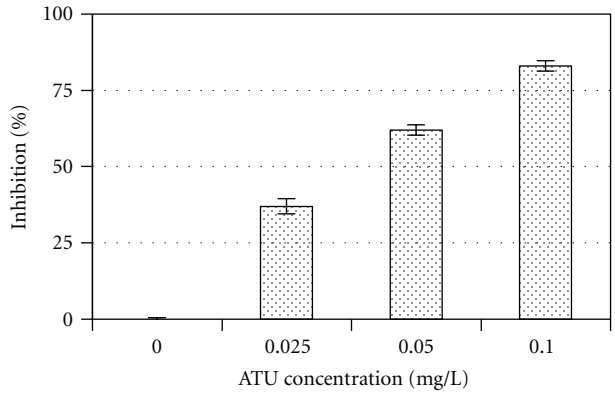

FIGURE 5: Nitrification inhibition as a function of ATU concentration. Error bars represent the standard error ($n = 2$).

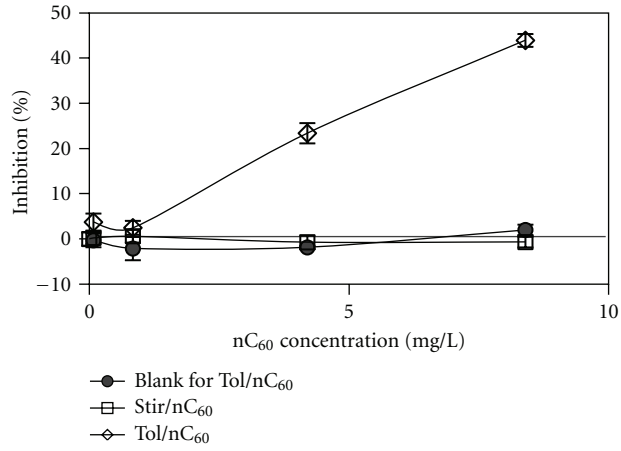

FIGURE 6: : Nitrification inhibition of nC_{60} as a function of preparation method and exposure concentration. Error bars represent the standard error ($n = 2$).

formation (NO_3^- and NO_2^-) between the control and the nC_{60} exposure test after 3 h. The equation is given as,

$$I\,(\%) = \frac{(N_c - N_f)}{(N_c - N_i)} \times 100, \quad (1)$$

where N_c (mg-N/L) is the concentration of oxidized N in the control flask after 3 h, N_f (mg-N /L) is the concentration of oxidized N in the flask containing nC_{60} after 3 h, N_i (mg-N/L) is the concentration of oxidized N in the flask containing the reference inhibitor of N allylthiourea (ATU). The EC_{20}, nC_{60} concentration with a reduction of oxidized N formation by 20%, was calculated using the SPSS probit regression analysis (SPSS, USA).

2.4. Microtox Test. The *V. fischeri* bioassay is also used for assessing the toxicity of compounds on the activated sludge [23, 24] which is based on the decrease in bioluminescence from the bacterium due to the exposure to the toxicants. The experiment was carried out using a Model 500 luminometer (Azur Environmental, USA) in accordance with the Microtox acute toxicity procedure [25]. The reagent (freeze-dried *V. fischeri*) and the solutions (diluent, reconstitution, and osmotic adjusting solution) were purchased from Strategic Diagnostics Inc., USA. The EC_{20}, nC_{60} concentration with a reduction of bioluminescence by 20%, was calculated at each test with different exposure periods (5, 15, and 30 min) using the Microtox Omni Software (Strategic Diagnostics, USA). The phenol was used for the quality control of this test.

2.5. nC_{60} Aggregation in the Incubation Medium for the Toxicity Test. The aggregation of nC_{60} as a function of incubation time was determined using the same medium of the toxicity tests. For the nitrification inhibition test, the nC_{60} size in medium (2 mM $(NH_4)_2SO_4$, 6 mM $NaHCO_3$ and 2 mM KH_2PO_4 buffer) was measured after 5 min (minimum time for one measurement) and 3 h. And for the Microtox

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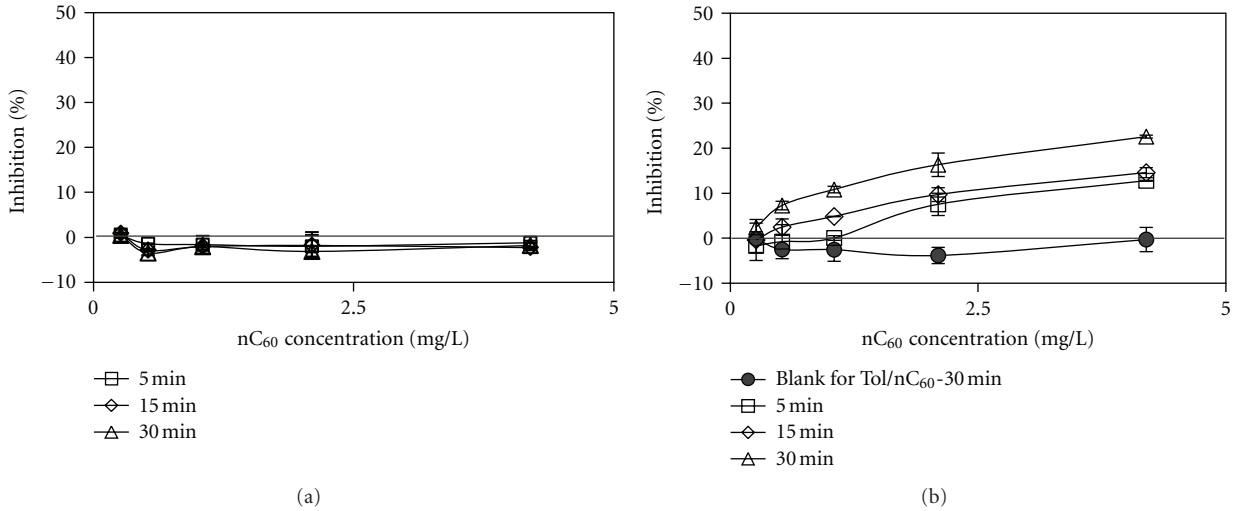

Figure 7: Bioluminescence inhibition of Stir/nC_{60} (a) and Tol/nC_{60} (b) as a function of exposure time and concentration. The Error bars represent the standard error ($n = 2$).

test, the nC_{60} size in 2% NaCl solution was measured after 5, 15, and 30 min.

2.6. Analysis. The NH_4^+, NO_3^-, and NO_2^- concentrations were determined based on the standard methods [26]. The UV absorbance of the stock C_{60} toluene solution and nC_{60} suspension in water was measured using a UV-2500 spectrophotometer (Shimadzu Scientific Instruments, Japan). nC_{60} size and its distribution were determined by the dynamic light scattering using a Zetasizer Nano ZS equipped with a 633 nm laser source and a detection angle of 173°C (Malvern Instruments, UK). The same instrument was used to measure the electrophoretic mobility which was subsequently calculated into Zeta potential using the Smoluchowski equation. All the tests in this study were conducted in duplicate.

3. Results and Discussion

3.1. Characterization of Prepared nC_{60}. Figure 1 shows the UV-visible absorption spectra of Stir/nC_{60} and Tol/nC_{60}. Both nC_{60} show the absorption peaks at 260 and 350 nm for the electronic structure of molecular C_{60} cage [27] and a broad absorption band at 450–550 nm for the aggregated C_{60}-C_{60} interactions [6]. Similar findings have also been reported for nC_{60} prepared via tetrahydrofuran (THF) [28]. However, the ratio of absorbance at 260, 350, and 450 nm varied with the nC_{60} types suggesting the difference in the structure and composition of nC_{60} aggregates [29].

Two types of nC_{60} demonstrated very similar size distribution with an average size of 154 and 144 nm for Stir/nC_{60} and Tol/nC_{60}, respectively, as shown in Figure 2. Both nC_{60} were negatively charged with no significant difference at pH 5.6 (Figure 3), which is in agreement with the reported results of nC_{60} prepared using similar methodologies [29].

3.2. Aggregation of nC_{60} in Toxicity Test Medium. The aggregate size is an essential information when assessing the toxicity of nanoparticles because of proved correlation [7, 30]. Figure 4(a) shows the nC_{60} size with time in medium for nitrification inhibition test. After the incubation time of 3 h the aggregate remained stable with only several nm of increase in size for both nC_{60}. It can be explained by the low ionic strength of this medium (~15.8 mM) which was much lower than the reported threshold destabilization concentration of ~120 mM for the nC_{60} [31]. In contrast, the obvious increase in size was found in medium with high ionic strength (~342 mM) for the Microtox test (Figure 4(b)). Compared to the initial sizes in pure water, the size in medium increased by 31, 56, and 114% for Stir/nC_{60} and 55, 97, and 142% for Tol/nC_{60} after 5, 15, and 30 min. In addition, the aggregation rate of Stir/nC_{60} was slower than that of Tol/nC_{60} presumably due to the difference in the structure and chemistry of nC_{60} aggregate. Brant et al. found the hydrophobicity of Stir/nC_{60} was lower than the nC_{60} prepared via the organic solvent, such as, toluene and THF [29].

3.3. Effect of nC_{60} on Nitrification Activity. The sludge nitrification activity and test performance were confirmed using the ATU. The EC_{50} was calculated to be 0.040 mg/L from the data (Figure 5) which was close to the published value of 0.025 mg/L [32]. Figure 6 shows the percent nitrification inhibition by two types of nC_{60} at varying concentrations after 3 h, as well as the blank sample for Tol/nC_{60}. For the Stir/nC_{60}, no nitrification inhibition was observed up to 8.4 mg/L indicating its low toxicity on nitrifying activated sludge. Previous studies also presented similar results that no significant impact was identified on the microbial community structure in aerobic soil [33] and anaerobic sludge [16]. In the case of the Tol/nC_{60}, no obvious effect was found in the blank sample (less than 2%).

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But ~40% nitrification was inhibited at 8.40 mg/L, and the EC_{20} was calculated to 4.89 mg/L for Tol/nC_{60}. It clearly showed that the nC_{60} toxicity depended on the preparation method. Zhu et al. [8] compared the toxicity of Stir/nC_{60} and nC_{60} produced via THF on *Daphnia magna* and founded EC_{50} (48 h) for the latter was at least one order of magnitude (0.8 mg/L) less than that for Stir/nC_{60} (>35 mg/L).

3.4. Effect of nC_{60} on Bioluminescent Bacteria. Figure 7(a) shows the percent inhibition due to the exposure to Stir/nC_{60} at different concentrations. No inhibition was observed at the concentration up to 4.2 mg/L for all the incubation time. In contrast, the Tol/nC_{60} showed obvious inhibition at >1.05 mg/L, and the toxicity increased with the incubation time (Figure 7(b)). No inhibition was also observed for the blank sample up to 30 min. The EC_{20} was calculated to 4.96, 4.98, and 3.44 mg/L for 5, 15, and 30 min, respectively. Both the facts above confirmed the low toxicity of Stir/nC_{60} and the toxicity dependent on the preparation methods. The result is consistent with that obtained from the nitrification inhibition test.

4. Conclusions

The prepared Stir/nC_{60} and Tol/nC_{60} showed similar surface properties, such as, the size distribution, zeta potential, and UV-vis absorption spectra. However, two types of nC_{60} presented different aggregation rates in test medium during the incubation periods. Both the nitrification inhibition and Microtox test showed that the nC_{60} toxicity was greatly affected by the preparation method. Stir/nC_{60} presented no significant toxicity to the nitrification sludge and bioluminescent bacteria at maximum concentration studied. In contrast, the EC_{20} of Tol/nC_{60} was obtained to be 4.89 mg/L (3 h) for the nitrification inhibition and 3.44 mg/L (30 min) for Microtox test, respectively.

Conflict of Interests

The authors declare that there is no conflict of interests.

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